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Purification and characterization of an extracellular lipase from a novel strain *Penicillium* sp. DS-39 (DSM 23773)

Dharmendra S. Dheeman[a,*], Sanjay Antony-Babu[b,c], Jesús M. Frías[a], Gary T.M. Henehan[a]

[a] School of Food Science & Environmental Health, Dublin Institute of Technology (DIT), Cathal Brugha Street, Dublin 1, Ireland
[b] Institute for Research on Environment & Sustainability, School of Biology, Newcastle University, Devonshire Building, NE17RU Newcastle, UK
[c] Institut National de la Recherche Agronomique (INRA), UMR 1136 'Interactions Arbres Micro-organismes', Centre INRA de Nancy, 54280 Champenoux, France

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ABSTRACT

A newly isolated fungal strain, *Penicillium* sp. DS-39 (DSM 23773), was found to produce an inducible extracellular lipase when grown in a medium containing 1.0% (v/v) olive oil. Maximum lipase activity was obtained after 120 h of incubation at 28 °C. The lipase was purified 129-fold with a final specific activity of 308.73 IU/mg. The molecular weight of the homogeneous lipase was 43 kDa as determined by SDS-PAGE. It was optimally active at pH 5.5 and 45 °C. The lipase was most active on triolein and exhibited a broad substrate range with a preference for triacylglycerols containing long chain unsaturated fatty acids. It showed no regio-specificity for the ester bond in triolein. It was activated by Ca^{2+} and Mn^{2+}, while significant inhibition was observed with Hg^{2+} and Zn^{2+}. The lipase showed significant stability and activation in the presence of organic solvents with log $P \geq 2.0$. These features render *Penicillium* sp. DS-39 lipase (PEL) a potential biocatalyst for applications such as biodiesel production, enzymatic restructuring, by interesterification of different oils and fats, and biodegradation of oil spills in the environment.

1. Introduction

Lipases (EC 3.1.1.3) are ubiquitous hydrolytic enzymes that catalyze the breakdown of fats and oils into free fatty acids, monoacylglycerols, diacylglycerols and glycerol and operate at the interface of emulsified lipid substrates [1]. They constitute one of the most important groups of industrial enzymes due to their unique ability to hydrolyze fatty acid ester bonds in aqueous environments and synthesize them in non-aqueous medium [2]. Lipases have proven to be efficient and selective catalysts in many industrial applications, most of them involving the modification of fats and oils to high added-value lipid-based products [3]. Industrial applications to date range from use in detergent and paper manufacturing to the production of structured lipids, bioesters and biosurfactants based on carbohydrate esters [4–6]. These industrial applications have stimulated interest in isolation of new lipases from novel sources.

A considerable number of bacterial and fungal lipases have been commercially produced, the latter being preferable because fungi generally produce extracellular enzymes, which facilitates easy recovery of the enzyme from the fermentation broth [7]. Among fungi, the genus *Penicillium* contains many lipase producers [8–11]. *Penicillia* are widely used as a source of lipases [12]. The presence of lipolytic enzymes in moulds of the genus *Penicillium* are employed in the dairy industry, since these lipases play a major role in developing characteristic flavors in ripened cheeses [13]. Also lipases from *Penicillia* are useful in a number of bioconversions of industrial importance [14–20].

We have isolated a lipase producing fungal strain identified as *Penicillium* sp. DS-39 through 18S rRNA gene sequencing. In this study, we report production, purification and characterization of an inducible extracellular lipase secreted by *Penicillium* sp. DS-39. In addition, the purified lipase has been shown to be of potential use in non-aqueous biocatalysis owing to its broad substrate range coupled with its significant stability and activation in non-polar organic solvents.

2. Experimental

2.1. Materials

Analytical reagent grade chemicals were obtained from commercial sources at the purest grade available. Unless otherwise

Abbreviations: PEL, *Penicillium* sp. DS-39 lipase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DEAE, diethylaminoethyl; MUF-oleate, 4-methylumbelliferyl oleate; PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; EDTA, *N,N,N′,N′*-ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride.

* Corresponding author. Tel.: +353 14024359; fax: +353 14024459.
E-mail addresses: dharmendra.singh@dit.ie, dheeman@gmail.com (D.S. Dheeman).

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mentioned, all chemicals were purchased from Sigma–Aldrich Ireland Ltd. (Dublin, Ireland).

2.2. Isolation and identification

The fungal strain used in this study was isolated by screening a soil sample collected from Phoenix Park, Dublin, Ireland. The soil sample (1 g) was suspended in 9 ml of sterile one quarter strength Ringer's solution (Oxoid, Basingstoke, UK) and serial dilutions were made. Aliquots (0.1 ml) of appropriate dilutions were surface plated on tributyrin agar. Plates were then incubated at 28 °C and periodically examined for 4–5 days. The fungal strain DS-39 was identified as a lipase producer on tributyrin agar through zone of clearance. The micromorphology of the isolate was studied by viewing lacto-phenol cotton blue wet mount preparations. Based on the morphological data, the isolate was assigned to the genus *Penicillium*. Confirmation of the assigned taxon was carried out by 18S rRNA gene sequence analysis. PCR amplification of this gene was carried out using primers NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS4 (5′-CTTCCGTCAATTCCTTTAAG-3′). The amplified product was sequenced using ABI 3730 at Geneius Laboratories Ltd., Newcastle, UK. Partial 18S rRNA gene sequence thus obtained was submitted to GenBank database at NCBI (GeneBank accession No. HM579932). This sequence was submitted as a query to BLASTn search at NCBI server to identify the nearest neighbor sequences. A phylogenetic tree was constructed by neighbor-joining algorithm using maximum composite likelihood method, with18S rRNA gene of *Septofusidium herbarum* CBS 265.58 (GenBank accession No. AY526480) as an outgroup and bootstrap based on 1000 replicates, in MEGA 4.1 [21]. The identified strain *Penicillium* sp. DS-39 was deposited in the culture collection at the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, under the accession No. DSM 23773.

2.3. Microorganism and culture conditions

The strain was maintained on potato dextrose agar (PDA, Scharlau Chemie, Spain) at 4 °C. For spore production PDA was used. After 5 days of incubation at room temperature (~20 °C), spores were collected using a sterile aqueous solution containing 0.02% (v/v) Tween 80 and 0.8% (w/v) NaCl. The liquid medium for lipase production consisted of (g/l): bacto-peptone, 5.0; yeast extract, 1.0; $NaNO_3$, 0.5; KCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; KH_2PO_4, 2.0 and olive oil, 10.0. The pH was adjusted to 7.0 ± 0.2 with 1.0 M NaOH. The cultivation was performed in 1 l Erlenmeyer flask containing 0.5 l medium at 28 °C, under orbital agitation (180 rpm) for 168 h using an inoculum of 1.8 ml spore suspension (10^8 spores/ml). The cultures were filtered (Whatman® No. 1 filter paper) at different time intervals and the mycelia harvested were dried (90 °C, 48 h) to constant weight for growth studies. The culture filtrate obtained after 120 h incubation was passed through a 0.22 μm pore size filter (Millipore® Stericup™ filter unit) and the "cell-free" filtrate thus obtained was used as a source of extracellular lipase.

2.4. Lipase assay

Lipase activity was assayed using *p*-nitrophenyl palmitate (*p*-NPP) as substrate according to Winkler and Stuckmann [22] with some modifications as described [23]. The assay was typically run for 10 min at 45 °C before termination by addition of 2 ml of 0.2 M Na_2CO_3. Liberated *p*-nitrophenol was determined at 410 nm ($\varepsilon_{410\,nm}$ = 0.0169/μM cm) using a UNICAM UV2 2000E UV-VIS spectrophotometer (Cambridge, UK). Appropriate controls (blanks without enzyme) were used to subtract the absorbance corresponding to the reaction mixture other than that produced by the specific hydrolysis of *p*-NPP. One international unit (IU) was defined as the amount of enzyme needed to liberate 1 μmol of *p*-nitrophenol per minute under the assay conditions.

2.5. Protein assay

Protein concentrations were determined by Bradford protein microassay [24] using bovine serum albumin (BSA) as a standard. During chromatographic purification steps, protein concentrations were monitored by measuring the absorbance of fractions at 280 nm ($A_{280\,nm}$).

2.6. Purification of PEL

The cell-free culture filtrate, from a 120 h-old culture grown in an olive oil based medium, was incubated for 20 min at 55 °C. After rapid cooling, insoluble denatured proteins were removed by centrifugation at 15,000 × *g* for 30 min at 4 °C. The resulting supernatant, containing PEL activity, was loaded on a DEAE-cellulose column (1.5 × 20 cm) equilibrated with buffer A (20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 2 mM benzamidine). Under these conditions, PEL was not adsorbed onto the support and was eluted during washing by buffer A. PEL-active fractions eluted from DEAE-cellulose were pooled and subjected to ammonium sulfate protein precipitation at 70% saturation, with constant stirring for 30 min at 4 °C, followed by centrifugation at 15,000 × *g* for 30 min at 4 °C. The pellet obtained was resuspended in buffer A (10 ml) and the insoluble material was removed by centrifugation at 15,000 × *g* for 30 min at 4 °C. The supernatant obtained was concentrated (Centricon Plus-70, Biomax 5000 MWCO, Millipore, UK) and loaded on a Sephacryl® 100-HR column (1.0 × 100 cm) equilibrated with buffer A. Elution of PEL from Sephacryl® 100-HR was performed with buffer A at a flow rate of 10 ml/h. The fractions, containing PEL activity, eluting between 1.2 and 1.3 void volumes, were pooled and applied to a Q Sepharose® HP column (2.5 × 20 cm) equilibrated with buffer B (20 mM Tris–HCl, pH 8.0, 10 mM NaCl, 1 mM benzamidine). Unbound proteins from Q Sepharose® HP were washed out with buffer B and the adsorbed proteins were eluted with a linear gradient of NaCl. During gradient elution with 10–250 mM NaCl, PEL emerged at 150 mM NaCl. The fractions, containing PEL activity, were pooled, concentrated (Centricon Plus-70, Biomax 5000 MWCO, Millipore, UK) and loaded on a Sephacryl® 100-HR column (1.0 × 100 cm) equilibrated with buffer A. Elution of PEL from Sephacryl® 100-HR was performed with buffer A at a flow rate of 10 ml/h. The fractions containing PEL activity were eluted as a single protein peak at 1.3 void volumes. PEL-active fractions were pooled and purity of PEL was determined by SDS-PAGE.

2.7. Gel electrophoresis and zymography

SDS-PAGE was carried out in 12.5% (w/v) gels according to Laemmli's method at room temperature (ATTO AE-6450, Tokyo, Japan) as previously reported by Dheeman et al. [23]. For activity staining zymographic analysis was performed using MUF-oleate as a substrate essentially as described by Prim et al. [25].

2.8. Deglycosylation

In order to analyze for the presence of carbohydrate moieties in the purified PEL, deglycosylation was carried out using endoglycosidase (Endo H, NEB, UK) according to the manufacturer's instructions. Twenty microliters of purified PEL (5.0 μg) was incubated in 0.05% SDS and 0.1% β-mercaptoethanol at 100 °C for 10 min. Then 1 μl of Endo H and 2 μl 50 mM sodium citrate (pH 5.5) was added to the reaction mixture and incubated at 37 °C for